REMARKS

Favorable reconsideration is respectfully requested in view of the foregoing amendments and the following remarks.

I. CLAIM STATUS & AMENDMENTS

Claims 1-26 were pending in this application when last examined.

Claims 1-15, 18 and 19 were examined on the merits and stand rejected.

Please clarify the status of the withdrawn claims. In item (4a) on page 1 of the Office Action, it was indicated that claims 1, 16 and 20-26 were withdrawn. However, in item (2) on page 2, it was indicated that claims 16 and 20-26 were withdrawn. Moreover, kindly note that claim 1 should not have been included in the list of withdrawn claims for it is part of elected Group I and was included in the prior art rejections. Further, it appears that claim 17 should have been included in the list of withdrawn claims for it was not part of elected Group I. Thus, the correct withdrawn claims seem to be claims 16, 17 and 20-26. Please confirm and/or clarify.

Claim 1 is amended to clarify that the claimed methods are high-throughput methods, which allow simultaneous production of a plurality of monoclonal antibodies against different candidate antigens. Claim is further amended to specify that immunization occurs with a plurality of candidate antigens. Support can be found in the disclosure, for example, at page 1, lines 1-3, page 2, lines 20-27, page 3, lines 20-29, page 4, lines 20-27, page 13, line 27 to page 14, line 4, and original claims 1, 3 and 14. Further support for immunizing more than one animal is found in the disclosure, for example, at page 5, lines 26-30 and page 6, lines 7-8. Support for displaying the antigens on more than one chip is found throughout the specification, for example, on page 9, lines 18–20, lines 29 to page 10, line 2, and page 14, lines 1-4.

Claims 2, 4, 5, and 7-11 are amended to include a comma before "wherein" to better conform with US practice and English form.

Dependent claims 2, 4, 5, 6 and 8 are amended along the lines of claim 1 to reflect immunizing more than one animal. See above for the desired support.

Dependent claim 4 has been amended to correct its dependency and the reference to "one" antigen has been replaced by "two" antigens in view of the amendment to claim 1. See above for the desired support.

Dependent claim 9 is amended along the lines of claim 1 to reflect the plural form of immortalized cell lines. See above for the desired support.

Dependent claims 10 and 13 are amended along the lines of claim 1 to reflect displaying the antigens on more than one chip See above for the desired support.

Claims 18-19 are amended to replace the reference to "antigens derived from a different source" to "antigens obtained from different proteins." Support can be found in the disclosure, for example, at page 15, lines 1-7.

Claims 3, 14 and 15 are cancelled without prejudice or disclaimer thereto. Applicants reserve the right to file a continuation or divisional application on any cancelled subject matter.

Claim 27 is newly added. Support can be found in the disclosure, for example, at page 14, lines 1-4.

No new matter has been added

Claims 1, 2, 4-13 and 16-27 are pending upon entry of this amendment.

II. CLAIM FOR FOREIGN PRIORITY

Kindly acknowledge the Applicants' foreign priority claim, including receipt of certified copies of the foreign priority documents.

III. INFORMATION DISCLOSURE STATEMENT

Applicants did not receive an Examiner-initialed copy of the PTO-1449 Form (IDS filed October 14, 2004), indicating that the references cited therein have been officially considered and

made of record by the Office. Thus, kindly consider the cited references and return an Examiner-initialed PTO-1449 Form indicating such.

IV. CLAIM OBJECTION

In item 4 on page 2 of the Action, claim 1 was objected to on the basis that step (e) recites "selecting as said monoclonal antibody". The present amendment overcomes this objection for reasons which are self-evident.

V. INDEFINITENESS REJECTION

In item 5 on pages 2-3 of the Action, claims 18 and 19 were rejected under 35 U.S.C. § 112, second paragraph, as indefinite for the recitation of "antigen being derived from a different source."

The present amendment overcomes this rejection. In particular, claims 18-19 are amended to replace "antigens derived from a different source" with "antigens obtained from different proteins," thereby removing the "derived from" language. Support can be found in the disclosure, for example, at page 15, lines 1-7.

It is clear from this disclosure that the reference to candidate antigens "derived from different sources" is intended to mean that the antigens are obtained from different proteins, and that methods involving immunizing an animal or animals with fragments of the same protein are excluded from the invention.

Thus, the indefiniteness rejection of claims 18 and 19 under 35 U.S.C. § 112, second paragraph, is untenable and should be withdrawn.

VI. ANTICIPATION REJECTIONS

In item 7 on pages 4-5 of the Action, claims 1-9 and 13 were rejected under 35 U.S.C. § 102(b) as anticipated by Kucherlapati et al. (US 6,150,584).

This rejection is respectfully traversed as applied to the amended claims.

To anticipate a claim, a cited prior art reference must teach each and every element of the claimed invention. M.P.E.P. § 2131.01.

Amended claim 1 is directed to a high-throughput method for producing a plurality of monoclonal antibodies, each of which binds to a different candidate antigen, said method comprising the steps of: a) introducing a plurality of candidate antigens into an animal or animals; b) recovering antibody-producing cells from said animal or animals and rendering these cells into a single cell suspension; c) generating immortalized cell lines from said single cell suspension; d) screening the supernatant of said immortalized cell lines against a protein chip or protein chips on which the candidate antigens are displayed; and (e) selecting monoclonal antibodies that bind to said candidate antigens.

Accordingly, amended claim 1 clarifies that the methods of the invention are high-throughput methods that allow the simultaneous production of a plurality of monoclonal antibodies against a plurality of different antigens by immunizing animals with a plurality of antigens. Monoclonal antibodies against specific antigens are identified by spotting supernatants from antibody-producing hybridoma cells onto a protein chip bearing the antigens.

Kucherlapati et al. fail to disclose or suggest immunization of animals with a plurality of antigens to simultaneously produce monoclonal antibodies against a plurality of antigens. Further, Kucherlapati et al. does not disclose or suggest the identification of monoclonal antibodies against multiple antigens using a protein chip.

Instead, Kucherlapati et al. describes a transgenic animal, in particular, a transgenic mouse, that contains human antibody transgenes that allow it to produce human antibodies. Kucherlapati et al. describe the use of this transgenic mouse in the production of human monoclonal antibodies against an antigen. A single antigen is used to immunize the transgenic mouse (column 4, lines 41-50), and B cells from the immunized mouse are used to generate hybridomas producing monoclonal antibodies against this single antigen using Kohler &

Milstein's standard method (column 7). Monoclonal antibodies binding to the antigen are identified by screening using a sandwich ELISA (column 7).

All of the examples in Kucherlapati et al. describe immunization of the animal with a single antigen followed by generation of hybridomas and identification of monoclonal antibodies binding to the antigen using standard ELISAs. In particular, Example 9 (referred to by the Examiner) discloses immunization of the mouse with recombinant IL-8 (column 18, lines 15-20), isolation of spleen cells and generation of hybridomas (column 18, lines 35-50) and identification by ELISA of hybridomas producing monoclonal antibodies that bound IL-8 (column 18, lines 55-62).

Accordingly, it is clear that Kucherlapati et al. does not describe immunization of animals with a plurality of antigens in order to produce monoclonal antibodies against a plurality of antigens simultaneously. Also, Kucherlapati et al does not describe the identification of monoclonal antibodies against multiple antigens using a protein chip.

In fact, Example 9 of Kucherlapati et al. describes the use of a protein chip to analyze the kinetic properties of anti-IL-8 antibodies that have already been identified by ELISA. Moreover, recombinant IL-8 is the only antigen on the protein chip described in Kucherlapati et al. Accordingly, Kucherlapati et al. does not disclose or suggest the use of protein chips displaying multiple antigens, nor the use of such chips to screen hybridoma supernatants for monoclonal antibodies that bind to multiple antigens as in the claimed invention.

For these reasons, the invention of amended claim 1 is novel over Kucherlapati et al. Likewise, the invention of dependent claims 2 and 4-13 (dependent on claim 1) are also novel over Kucherlapati et al.

In addition, please note that claims 18-19 and new claim 27 are also novel over Kucherlapati et al., since the reference does not disclose or suggest immunization with a plurality of purified antigens as in claim 18, nor the screening of immortalized cell lines against protein chips on which multiple antigens are displayed as in claim 27.

For these reasons, it is respectfully submitted that Kucherlapati et al. cannot anticipate the claimed invention, because the reference fails to disclose or suggest each and every element of the invention.

Therefore, the rejection of claims 1-9 and 13 under 35 U.S.C. §102 (b) over Kucherlapati et al. is untenable and should be withdrawn.

In item 8 on pages 5-6 of the Action, claims 18 and 19 were rejected under 35 U.S.C. § 102(b) as anticipated by Mather et al. (WO 2000/037503).

This rejection is respectfully traversed as applied to the amended claims.

Amended claim 18 is directed to a method for producing a plurality of monoclonal antibodies, each of which binds to a different purified candidate antigen, comprising introducing a plurality of purified candidate antigens into an animal, each purified candidate antigen being obtained from a different protein.

Amended claim 19 is directed to a method for producing a plurality of monoclonal antibodies, each of which binds to a different purified candidate antigen, comprising introducing a plurality of purified candidate antigens into an animal, each purified candidate antigen being obtained from a different protein, which further comprises any of the steps recited in claim 1.

Accordingly, claims 18-19 require immunization with "purified candidate antigens". The specification at page 3, lines 11-19 defines "purified candidate antigen" as meaning the antigen is a homogenous preparation of antigen that is substantially free from other components. The specification at page 3 also specifies that the term "purified candidate antigen" does not include antigens contaminated with cellular debris.

Mather et al. fail to disclose or suggest immunization of mammals with purified antigens.

Instead, Mather et al. describe a method for generating a population of monoclonal antibodies capable of binding to antigens representative of a particular cell type by immunizing a mammal with "a plurality of viable and intact cells of said cell type". See paragraph 2 of the

Summary of the Invention of Mather et al. Accordingly, Mather et al does not describe immunization of mammals with purified antigens. Indeed, it is clear from paragraph 3 of the Background of the Invention of the reference, that Mather et al. consider extraction of purified antigens for use as immunogens to be disadvantageous. In this sense, Mather et al. teach away from the invention of claims 18 and 19.

Thus, it is clear that Mather et al. fail to disclose or suggest immunization of mammals with purified antigens. For this reason, Mather et al. cannot anticipate the claimed invention, for the reference fails to disclose or suggest each and every element of the claimed invention. Accordingly, it is clear that claims 18 and 19 do not encompass immunization with cells as described in Mather et al.

Claims 18 and 19 are thus novel over Mather et al.

In addition, it is noted that claims 1, 2 and 4-13 are also novel over Mather et al., since they also require immunization with a plurality of "purified" candidate antigens.

New claim 27 is also novel over Mather et al., since Mather et al. does not describe generating immortalized cell lines from a single suspension of antibody-producing cells that produce antibodies against a plurality of antigens, nor screening the supernatant of these immortalized cell lines against protein chips displaying multiple antigens.

In view of the above, the rejection of claims 18 and 19 under 35 U.S.C. § 102(b) as anticipated by Mather et al. is untenable and should be withdrawn.

VII. OBVIOUSNESS REJECTION

In item 11 on pages 8-13 of the Action, claims 1-15, 18 and 19 were rejected under 35 U.S.C. § 103(a) as obvious over Kucherlapati et al. in view of Mather et al. in view of Rava et al. (US 6,720,149, in view of Kessler et al. (US 2003/0044849).

This rejection is respectfully traversed as applied to the amended claims.

To establish obviousness, three criteria must be met. First, the prior art references must teach or suggest each and every element of the claimed invention. M.P.E.P. § 2143.03. Second, there must be some suggestion or motivation in the references to either modify or combine the reference teachings to arrive at the claimed invention. M.P.E.P. § 2143.01. Third, the prior art must provide a reasonable expectation of success. M.P.E.P. § 2143.02.

The deficiencies in the disclosures of Kucherlapati et al. and Mather et al. are discussed above and reiterated herein. Again, neither reference discloses or suggests immunizing an animal or animals with a plurality of purified candidate antigens to produce monoclonal antibodies against a plurality of antigens simultaneously. Nor do they disclose or suggest screening of monoclonal antibodies against a plurality of antigens using protein chips, let alone a high-throughput method of monoclonal antibody production combining both of these additional features.

For these reasons, Kucherlapati et al. and Mather et al. do not disclose or suggest each and every element of the claimed invention.

Furthermore, it is again noted that Mather et al. teaches away from the claimed invention. As discussed above, Mather et al. consider extraction of purified antigens for use as immunogens to be disadvantageous. See paragraph 3 of the Background of the Invention of Mather et al. In this sense, Mather et al. teach away from the present invention, which requires purified antigen.

Moreover, it is well established that there is no motivation to combine/modify references where the cited references teach away from the combination. The prior art must be considered in its entirety and that references cannot be combined where the references teach away from their combination. See M.P.E.P. § 2145 X, D, 2. A reference can be said to teach away when a person of ordinary skill in the art, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path taken by the applicant or if it suggests that the line of development flowing from the reference's disclosure is unlikely to be productive of the result sought by the applicant.

In view of the teaching away in Mather al., the cited references cannot be combined/modified to arrive at the claimed invention. Accordingly, there would be no reasonable expectation of success of combining/modifying the cited art teachings to arrive at the use of purified antigens as immunogens as in the present invention, because Mather al. discloses that it would disadvantageous to do so.

The secondary references of Rava et al. and Kessler et al. fail to remedy the above-noted deficiencies of Kucherlapati et al. and Mather et al.

Rava et al. describe a biological chip plate for conducting multiple biological assays. The plate contains a plurality of wells, each well containing a chip having a molecular probe array. The focus of Rava et al. is on the production of plates containing DNA arrays for use in detecting DNA or RNA molecules. The only reference to antibodies in Rava et al. is found in column 3, last line, which indicates that chips may be used to detect monoclonal antibodies.

However, there is no discussion at all in Rava et al. of the production of monoclonal antibodies. There is also no suggestion in Rava et al. that a plurality of antigens could be displayed on chips simultaneously to detect monoclonal antibodies that bind to a plurality of different antigens. Thus, it is clear that Rava et al. fails to remedy the above-noted deficiencies of Kucherlapati et al. and Mather et al.

Kessler et al. relates to the problem of identifying monoclonal antibodies that bind to a cell-associated antigen. Kessler et al. notes that it can be difficult to identify a monoclonal antibody that binds to a desired cell-associated antigen, because immunizing an animal with a cell carrying the desired antigen results in the production of monoclonal antibodies against other antigens on the cell surface, which will interact with the cell in subsequent screening steps. Kessler et al. propose the use of polyclonal antibody libraries (PALs) as a solution to this problem. A PAL is produced by immunizing an animal with a heterogenous preparation of antigens not having the cell-surface antigen of interest. The PAL can then be used to mask antigens on the target cell that are not of interest, making it easier to detect a monoclonal

antibody that binds to the cell surface antigen of interest. Accordingly, Kessler et al. is concerned with methods of producing antibodies by immunization with whole cells or heterogeneous mixtures. There is no teaching or suggestion in Kessler et al. that an animal or animals can be immunized with a plurality of purified antigens to produce monoclonal antibodies against a plurality of purified antigens. Nor is there any suggestion the resulting monoclonal antibodies be screened using protein chips displaying multiple antigens.

In summary, none of the references cited by the Office suggest immunizing an animal or animals with a plurality of purified candidate antigens in order to produce monoclonal antibodies against all of these antigens. In addition, none of the cited references suggest using protein chips displaying a plurality of antigens to screen for monoclonal antibodies that bind to these antigens. In fact, the presence of both of these steps in a single method results in enormous advantages over conventional methods of producing a single monoclonal antibody by immunizing an animal with a single antigen, producing hybridoma cells using Kohler & Milstein's method and identifying monoclonal antibodies against the antigen using an ELISA.

As evidence of these advantages, Applicants enclose a copy of a paper published by the inventors presenting additional data which have been obtained since the current application was filed (De Masi et al., Proteomics, Vol. 5, pp. 4070-4081, 2005).

As indicated in the paper De Masi et al., conventional methods of producing monoclonal antibodies by immunizing an animal with a single antigen, producing hybridoma cell lines and identifying a monoclonal antibody against the antigen using ELISAs result in the production of only about 20 high-affinity monoclonal antibodies against an antigen per year per person. In stark contrast, the manuscript describing the present invention discloses immunizing eight mice with 10 antigens each (80 antigens total), harvesting of the spleens 18 days later and screening for monoclonal antibodies against the antigens using protein chips. The paper discloses that the method of the invention allowed the inventors to perform the equivalent of 614,400 separate ELISA tests (which would require 1,600 x 384-well plates) in a 72-hour period. Furthermore,

only 5μg of each antigen was used in the screening procedure, as opposed to 200μg that is required to screen the same number of supernatants by ELISA. The entire process of producing monoclonal antibodies against 68 of the 80 antigens with which the mice were immunized took just 3 weeks.

Thus, the claimed methods enable the production and screening of monoclonal antibodies against large numbers of antigens simultaneously. This advantage alone represents huge progress over methods of generating and screening monoclonal antibodies against a single antigen, as disclosed for example, in Kucherlapati et al. The claimed methods enable huge numbers of hybridoma cell supernatants to be screened compared to prior art methods, making it more efficient at identifying high affinity antibodies. Most importantly, the methods claimed are simple and many times faster than conventional methods of the type disclosed in Kucherlapati et al. The methods claimed are also more economical due to the fact that they require hardly any antigen for use in the screening procedure.

There is no suggestion in any of the other documents cited by the Examiner that an improved method for producing and screening monoclonal antibodies which enables the simultaneous and efficient production of monoclonal antibodies against a plurality of candidate antigens could be obtained by immunizing an animal or animals with more than one purified antigen and by screening the monoclonal antibodies produced using protein chips. It might well have been obvious to the skilled person at the priority date that it was desirable to achieve the advantages provided by the methods of the invention but it would not have been obvious how to achieve them.

It is respectfully submitted that such advantageous and unexpected results are indicative of the nonobviousness of the present invention.

Kohler and Milstein's method of producing monoclonal antibodies disclosed in, for example Kucherlapati et al, had been known for over 25 years at the priority date. Protein chips had also been known before the priority date of the present application, as demonstrated by the

disclosure of Rava et al. In view of the importance of monoclonal antibodies in molecular biology, the need to provide an improved, high-throughput method of producing monoclonal antibodies was apparent well before the priority date. However, despite the fact that methods of producing monoclonal antibodies were known and the existence of protein chips, no-one had considered combining these two techniques to produce a high throughput method of simultaneously producing monoclonal antibodies against a plurality of antigens, as now claimed. This, in itself, suggests that the combination would not have been obvious to the skilled person at the priority date.

Furthermore, in the five years since the present application was filed, there has been no suggestion, other than by the inventors, of combining methods of producing monoclonal antibodies with methods of screening for monoclonal antibodies using protein chips. In this regard, Applicants enclose a copy of a paper by Chambers et al. (Nature Biotechnology, Vol. 21, No. 9, pp. 1088-1092, 2003), published well after the priority date for the present application, which attempts to solve the problem of developing a high-throughput method for producing polyclonal antibodies. Chambers et al. disclose the generation of polyclonal antibodies against multiple antigens by immunization with over 100 antigens and subsequent identification of the polyclonal antibodies using Western blots and ELISAs. Despite being published over a year after the priority date of the present application, this paper does not contemplate generating multiple monoclonal antibodies by immunization with multiple antigens or subsequent screening of antibodies on protein chips.

It is apparent that enormous advantages are obtained by using the method of the invention in comparison with the laborious prior art methods for producing monoclonal antibodies. It was of course hugely desirable in the prior art to achieve these advantages and if it had been obvious that they could be achieved by combining known methods of producing monoclonal antibodies with a step involving screening the antibodies using protein chips, the skilled person would surely have combined these methods previously. The Applicants found a way of doing

something, which people had wanted to do, but could not think how. They achieved this goal by using established techniques to do something which no one had previously thought of doing. The claimed methods are thus non-obvious over the prior art.

In view of the above, the 103(a) obviousness rejection of claims 1-15, 18 and 19 is untenable and should be withdrawn.

CONCLUSION

In view of the foregoing amendments and remarks, it is respectfully submitted that the present application is in condition for allowance and early notice to that effect is hereby requested.

If the Examiner has any comments or proposals for expediting prosecution, please contact the undersigned attorney at the telephone number below.

Respectfully submitted,

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ATTACHMENTS

1. De Masi et al., "High throughput production of mouse monoclonal antibodies using antigen microarrays", Proteomics, Vol. 5, pp. 4070-4081, 2005; and

2. Chambers et al., "High-level generation generation of polyclonal antibodies by genetic immunization", Nature Biotechnology, Vol. 21, No. 9, pp. 1088-1092, 2003.

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REGULAR ARTICLE

High throughput production of mouse monoclonal antibodies using antigen microarrays

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Recent advances in proteomics research underscore the increasing need for high-affinity monoclonal antibodies, which are still generated with lengthy, low-throughput antibody production techniques. Here we present a semi-automated, high-throughput method of hybridoma generation and identification. Monoclonal antibodies were raised to different targets in single batch runs of 6–10 wk using multiplexed immunisations, automated fusion and cell-culture, and a novel antigen-coated microarray-screening assay. In a large-scale experiment, where eight mice were immunized with ten antigens each, we generated monoclonal antibodies against 68 of the targets (85%), within 6 wk of the primary immunization.

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Hybridoma production / Microarrays / Monoclonal antibodies / Multiplexing

1 Introduction

Although analysis of gene expression patterns using nucleic acid microarrays has become a powerful tool in genomic- and proteomic-scale studies, the results of these screens do not detect the presence of the expressed gene product, namely the protein. Only monoclonal antibodies and related affinity reagents detect the functional unit itself, and are therefore powerful and desirable tools in this detection process. Mouse-derived monoclonal antibodies continue to be the affinity reagent of choice in proteomics analyses, but their production against novel targets remains restricted by high tissue culture load and low-throughput screening methods [1]. Other techniques more amenable to high-throughput production of high affinity detection reagents are still unable to yield high affinity antibodies without lengthy downstream manipulation [2–8]. We identified two obstacles to increasing the mAb production throughput level. The first is the number of tissue culture operations necessary for performing multiple fusions simultaneously using only one antigen per animal. The second is screening the many thousands of culture supernatants generated by large-scale production.

Here we present a semi-automated method of hybridoma generation using mice immunized with multiple antigens and a novel antigen microarray assay (AMA), which simultaneously detects antigen-specific binding and determines the isotype of the bound antibodies. When tested using 80 antigens, our system isolated monoclonal hybridomas against 68 of the targets (85%) in a single batch run within 6 wk of primary immunization.

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Abbreviation: AMA, Antigen microarray assay

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2 Materials and methods

2.1 Immunization

BALB/c mice were immunized at 21-day intervals using 25 μg of antigen and boosted with 10 μg of antigen in Imject Alum (Pierce, IL, USA) with the addition of 10 nmols CpG-DNA [9] per mouse (TCC ATG ACG TTC CTG ATG CT, TIB MOLBIOL, Genova, Italy). Mice were bled 10 days after each immunization and serum titre tested by ELISA. The mice were additionally boosted 4 days prior to fusion using the same adjuvant and route of immunization.

2.2 Fusion

The spleen was rendered into a single-cell suspension by mechanical disruption. The suspension was filtered into a 50-mL tube (BD Falcon) through 70-μm nylon cell strainers (BD Falcon). The tube was centrifuged at 100 × *g* for 10 min at room temperature (RT) and splenocytes resuspended in 5 mL Red Cell Lysis Buffer (Sigma, St. Louis, MO, USA) for 9 min at RT.

Hybridoma Medium HM20 (DMEM, 20% foetal bovine serum (Hyclone Defined), 10 mM L-glutamine, 50 μM Gentamicin) was added to a final volume of 50 mL and centrifuged for 10 min at RT with no brake. Supernatant solutions were aspirated to waste and cells resuspended in DMEM preheated to 37°C. Cells were washed twice more by steps of centrifugation and resuspension, and finally counted in a haemocytometer.

Separately, SP2 myeloma fusion partners (ATCC) were cultured for 5 days prior to fusion in HM20 and on the day of the fusion transferred to HM20/HCF/2xOPI (HM20 containing 10% Hybridoma Cloning Factor (Origen) and 2% OPI cloning supplement (Sigma)) for at least 1 h at 37°C in a 10% CO_2 humidified incubator. SP2 cells were washed three times in a similar fashion to the splenocytes and similarly counted. SP2 myelomas and spleen cells were mixed at a ratio of 1:5 (SP2:Spleen) and again centrifuged at 100 × *g* for 10 min with no brake.

The supernatant was entirely aspirated to waste and Polyethyleneglycol 1500 in 50% HEPES (PEG: Roche Molecular Biochemicals) pre-heated to 37°C was pipetted drop-wise over 1 min with agitation to ensure even mixing. The cell/PEG mixture was incubated for 1 min at 37°C with gentle agitation. One millilitre of DMEM was added drop-wise over 1 min at 37°C with agitation. The mixture was further incubated for 1 min at 37°C with gentle agitation. A further 1 mL of DMEM was similarly added over 1 min at 37°C with gentle agitation and incubated for a further minute. Seven millilitres of HM20 was added drop-wise over 3 min at 37°C with gentle agitation. The tube was then spun at 90 × *g* for 5 min with brake. The supernatant was aspirated to waste and the pellet resuspended in HM20/HCF/OPI/AH (HM20/HCF/OPI plus 10% Azaserine Hypoxanthine (Sigma)).

The post-fusion mixture was plated out into 20 96-well sterile plates (Nunc) at 100 μL/well and transferred to a humidified incubator (37°C, 10% CO_2).

On the third day after the fusion, the cells were fed with 100 μL HM20/HCF/OPI/AH. On day 7, culture supernatants were completely aspirated to waste and replaced with 150 μL of fresh HM20/HCF.

On day 11, 40 μL of each supernatant was transferred to 384-well plates (Greiner) as source plates for the microarray spotter.

2.3 Enzyme-linked immunoadsorbent assay (ELISA)

The 96-well plates (Maxisorp, Nunc) were coated with 4 μg/mL of antigen and incubated overnight at 4°C. The plates were washed in PBS 0.02% Tween-20 (PBST) and blocked with 3% BSA in PBS for 1 h at RT. Fifty microlitres of hybridoma supernatant was added to each well and incubated for 1 h at RT. After four washes in PBST, the plates were incubated for 1 h at RT with alkaline phosphatase conjugated anti-mouse secondary antibody, diluted 1:5000 in PBS (Jackson Dianova). Plates were washed in PBST and incubated with p-nitrophenyl phosphate (Sigma) for 10–15 min at RT. Reaction was stopped by adding 50 μL of 2 M NaOH and the OD was spectrophotometricly determined at 405 nm.

2.4 Microarray preparation

Aminosilane modified microscope slides (Aminosilane slides are generally available from EMBL-Heidelberg Genomics Core Facility (Genecore)) were homogeneously coated with 5 μg of antigen in 50 μL PBS using a 24 × 60 mm coverslip. Slides were incubated in a humid chamber at RT for 60 min, the coverslip removed and subjected to three 5-min washes in PBS. Slides were blocked in a 3% BSA solution in PBS for 60 min at RT. After five, 5-min washes in PBS, the slides were dried by centrifugation.

Hybridoma supernatants were spotted onto the slides using a MicroGrid II 600 arrayer, using 32 MicroSpot 2500 pins in an 8 × 4 array (Apogent Discoveries). Humidity and temperature were maintained at 40% and 24°C, respectively. Slides were left to incubate in the arrayer for a further 60 min. The microarrays were washed five times for 5 min in PBS and incubated with 40 μL of a mix of Cy3-conjugated goat anti-mouse IgG and Cy5-conjugated goat anti-mouse IgM (Jackson Immunoresearch, West Grove, PA, USA) both diluted 1:1000 in 3% BSA-10% Glycerol (60 min, RT, humid chamber). Microarrays were then washed twice for 5 min in PBST (0.2%), twice for 5 min in PBS and finally rinsed in ddH2O. Microarrays were dried by centrifugation and scanned in an LS400 Scanner (Tecan), using 633 and 543 nm lasers, respectively, for Cy5 and Cy3 excitation and 670 and 590 nm emission filters.

2.5 Data analysis

Image analysis was performed using the GenePix Pro 4.1 software package (Axon Instruments). Spots for which the diameter is not included in a fork of 80–150 μm or of bad quality (scratches, heavy background, dust, *etc.*) were ignored. For each remaining sample, we retrieved the median of the medians of the intensities of each group of replicates (MR). Each value was then normalised against the median value of all the MR of the chip [MR/(median of total MR)]. Samples showing a normalised value of less than two were considered negative. Values between 2 and 20 were considered putative positives, while all samples having a normalised value equal or over 20 were considered positives. Data analysis was performed using a proprietary software application, Hy-CAT (Hybridoma Chip Analysis Tool).

3 Results

3.1 Multiplexed immunisation and microarray screening of hybridomas

To minimize the overall tissue culture load we first investigated the potential of immunizing mice with more than one target antigen and generating and isolating hybridomas that secreted antibodies, which would specifically recognize each one of the target antigens.

We immunized a single Balb/c mouse with five antigens (Table 1a). The mouse was boosted at 3-wk intervals and serum titre levels against each of the antigens were monitored by ELISA 10 days after each boost. When serum titre levels had reached a level where all were reactive by ELISA at a dilution of 1:2500, we harvested the spleens and fused with SP2 myelomas to form hybridomas using the standard protocols [10]. The

Table 1. a) Antigens details. Details of the antigens used for this immunisation experiments are here described. b) Multiple antigen immunization and AMA analysis for the production and isolation of monoclonal antibodies. Five antigens were used for the immunisation of a single mouse and the generated hybridoma library was screened against each antigen with AMA. Monoclonal antibodies were detected and obtained for all antigens tested. The number of positive hybridomas and their isotypes breakdown (columns 2, 3 and 4) are shown. ELISA screens confirmed the positivity of all the IgG isotype monoclonals selected (column 5). As the ELISA screen was performed on AMA IgG positives only, no ELISA IgM data is available

1a:

Antigen	Protein's full name	Length of full protein	Size of antigen (kD or aa)	Expression method	Peptide/ protein	Species
Ago	PAZ domain of Argonaut 2	134.8 kDa	12.1 kDa	*E. coli*	Protein domain	*D. melanogaster*
Mago	Mago Nashi	17.3 kDa	17.3 kDa	*E. coli*	Protein	*D. melanogaster*
KetB4	Expressed fragment of Sls (2 Mda)	296 aa	34 kDa	*E. coli*	Protein	*D. melanogaster*
Tncf2	LiTnC1 (Troponin C isoform)	18 kDa	18 kDa	*E. coli*	Protein	*L. indicus*
PigMut4	Expressed fragment of Sls (2 Mda)	425 aa	48 kDa	*E. coli*	Protein	*D. melanogaster*

1b:

Antigen name	Total no. positive clones (AMA)	IgM secretors (AMA)	IgG secretors (AMA)	IgG secretors (ELISA)
Ago2	12	7	5	5
Mago JLY	17	8	9	9
KetB4	17	7	10	10
Tcnf2	29	9	20	20
Pig2Mut4	13	10	3	3

fusion was plated into 20 96-well plates. Culture supernatants were harvested 12 days after the fusion for screening.

To maximize the screening throughput we developed a novel antigen microarray assay (AMA) and screened the fusion as follows: five aminosilane treated glass slides were coated each with 5 μg of one of the target antigens (one antigen on each slide). All of the culture supernatants were then spotted as a microarray onto each of the antigen-coated slides. The slides were subsequently incubated with a simple mixture of Cy5-conjugated anti-mouse IgM and Cy3-conjugated anti-mouse pan-IgG (recognizing all mouse IgG isotypes). After washing and scanning in a microarray scanner, we detected both IgG and IgM antibodies that bound specifically to each target antigen, the colour of the fluorochrome indicating the isotype of the bound antibody (Fig. 1). The microarray scanning results were cross-referenced by comparing each of the microarrays with each other,

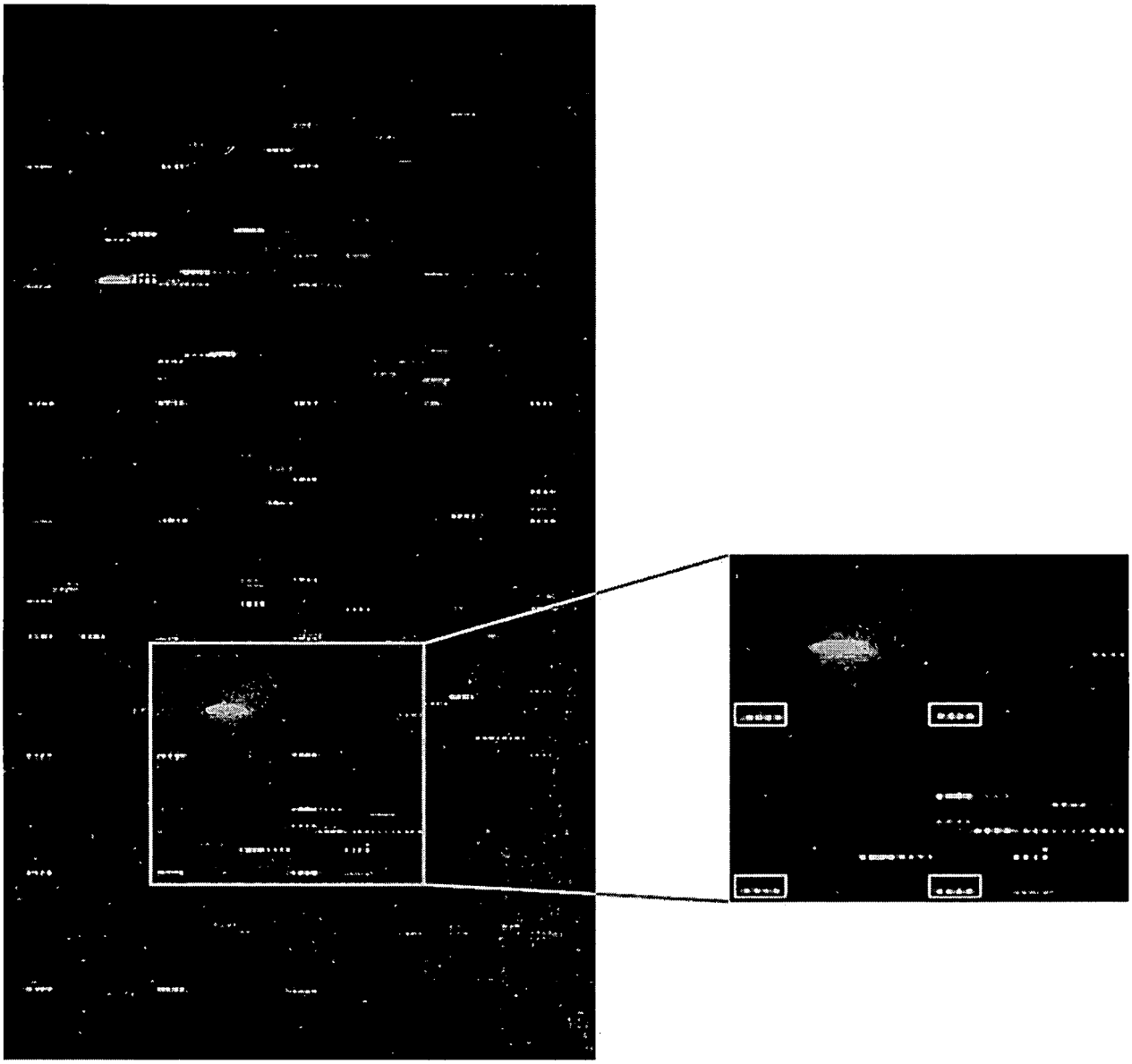

Figure 1. Scanned AMA chip. Scanned image of one AMA experiment. An aminosilane coated glass slide was coated with 5 μg of antigen and spotted with a library of 9,600 hybridoma supernatants. After a 60′ incubation in a humid chamber at room temperature, the array was hybridised with 50 μL of a solution containing Cy3-anti-mouse pan IgG and Cy5-anti-mouse IgM antibodies (diluted 1:1000). After washing, the array was scanned with a conventional microarray scanner. Red and green spots represent IgM and IgG monoclonal antibodies, respectively, specific for the coated antigen. Red boxes show the spots used as positive controls and sub-grid positioning aids. These consist of diluted blood samples from the immunised mice.

and cross-reactive clones recognizing more than one target antigen were eliminated. Clones positive by AMA were further tested by ELISA and confirmed to be positive as shown also in Table 1b.

3.2 AMA-ELISA correlation study

Since only clones shown positive by AMA were further tested by ELISA, we investigated whether both positive and negative AMA results correlated with the ELISA. Five mice were immunized with nine antigens (Table 2a) and hybridomas were produced and analyzed by AMA and ELISA as already described. KetB5, Ket94/95, HMG CoA and His-IPAPB analysis show correlation values ranging from 76.81% to 96.22%. Even though the number of positives for each of these antigens was variable, ELISA OD values for these antigens were all over 1.0. CSD SAP (3 ELISA positives/2 AMA positives), 4950 (3/2), and IPAPB Pep2 (2/1), showed very low ELISA OD values. However, the sample size in both assays for these antigens was too small to be statistically significant. Nineteen IPAPBmid positive hybridomas were positive by ELISA even though their ELISA ODs were still close to the 0.2 cut-off value but only 11 were positive by AMA (Table 2b).

The AMA results show a high correlation with ELISA but the dynamic range of the HybriChips is greater than that of the ELISA, making the detection of positive samples with ELISA intensities near background levels more difficult and subjective (Fig. 2).

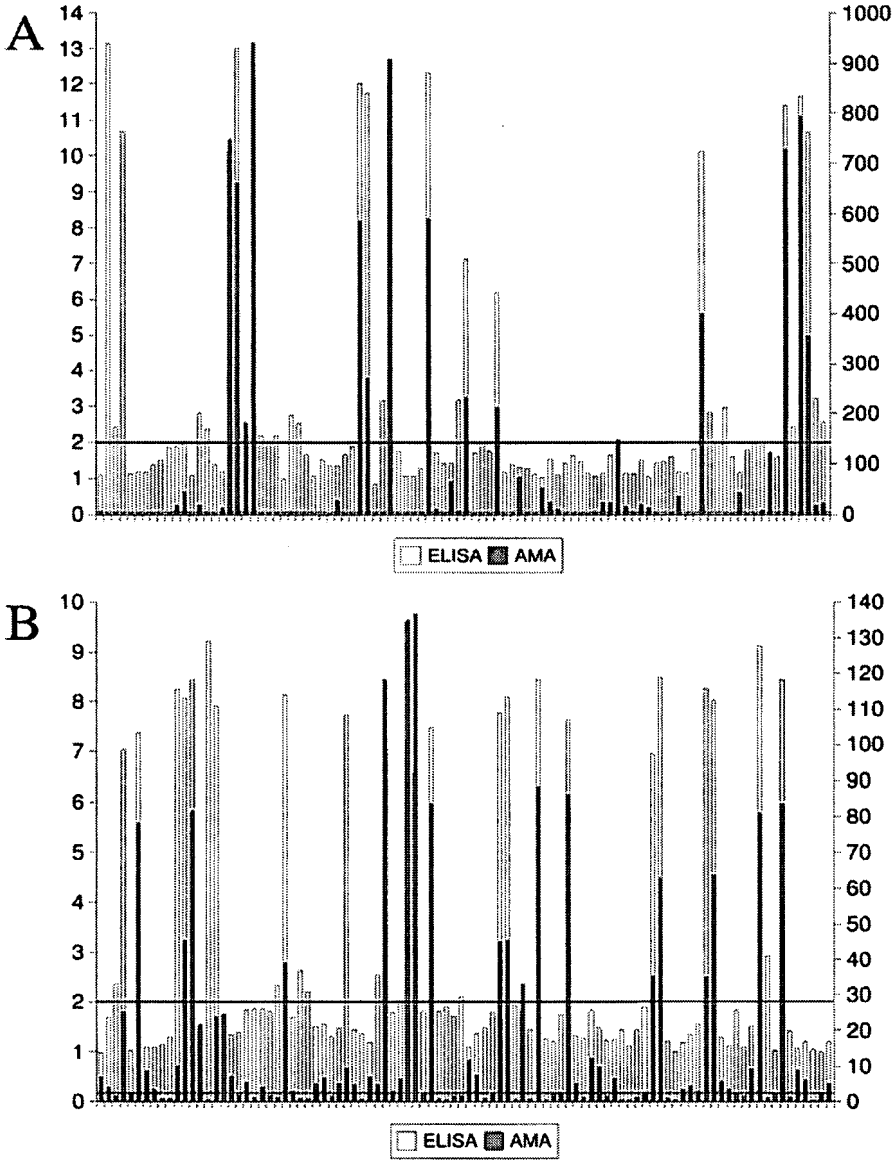

Figure 2. AMA-ELISA correlation. Correlation histograms for one representative 96-well plate for two of the antigens used for the trial, Ket94/95 (A) and KetB5 (B). Normalised values from the chip and the ELISA analysis were plotted in a sample-by-sample manner. HyrbiChip and ELISA normalised values are shown on the left and right axes respectively. The blue and red horizontal lines mark the "lower" normalised values required for a mAb to be considered positive on AMA and ELISA analysis, respectively.

Table 2. a) Antigens details. Details of the antigens used for this immunisation experiments are here described. No information about the 4950 antigen was obtained from the investigators. b) Correlation study between AMA and ELISA screening methods. Nine antigens were used to immunize five mice. Each hybridoma line was screened by both ELISA and AMA. The table shows the results for both assays, where the "ELISA value range" field shows the lower and higher OD values of the positive antibodies and the "AMA/ELISA matches" shows the number of antibodies positive by both assays. The "total correlation" value was calculated as the ratio between AMA and ELISA positives. "Novel" samples are those antibodies that were positive by AMA, but negative in the ELISA screen

2a:

Antigen	Protein's full name	Length of full protein	Size of antigen (kD or aa)	Peptide/ protein	Expression method	Species
GST Hupf	Hupf1	1,118 aa	1,398 aa	Protein	*E. coli*	*H. sapiens*
IPAPB-Mid	6xHis-inducible Poly(A) binding protein (middle fragment)	70 kDa	157 aa	Protein	*E. coli*	*H. sapiens*
His- IPABP	6xHis-inducible Poly(A) binding protein	70 kDa	71.6 kDa	Protein	*E. coli*	*H. sapiens*
IPABP-Pep2	inducible Poly(A) binding protein	70 kDa	15 aa	Peptide	Synthesised	*H. sapiens*
HMG CoA	HMG coenzyme A reductase	97.68 kDa	85 kDa	Fusion protein	*E. coli*	*H. sapiens*
CSD-SAP	Death inducer with SAP domain	1140 aa	63.5 kDa	Fusion protein	*E. coli*	*H. sapiens*
KetB5	Expressed fragment of Sls (2 Mda)	195 aa	22 kDa	Protein	*E. coli*	*D. melanogaster*

2b:

Antigen name	Mouse ID	ELISA positives	ELISA value range	AMA/ELISA matches	IgG	IgM	Total correlation	Novel
KetB5	540715	53	0.6052–1.6616	51	48	3	96.22%	4
Ket94	540715	15	0.7615–1.9997	13	13	0	86.67%	8
HMG CoA	1520	69	0.5094–1.9562	53	51	2	76.81%	0
CSD SAP	1520	3	0.2515–0.6053	2	1	1	66.00%	0
4950	540712	3	0.2508–0.4096	2	1	1	66.00%	1
GST-Hupf	540710	0	All <0.2	0	0	0	100.00%	0
His-IPAPB	540710	7	0.3042–1.2849	6	2	4	85.71%	0
IPAPB Pep2	540713	2	0.4048–0.4188	1	0	1	50.00%	0
IPAPB Mid	540713	19	0.1927–0.57	11	11	0	57.89%	0

3.3 Scale-up of the platform: 80 antigen trial

To test whether the AMA would be useful in a scaled up experiment we adapted and customized a commercially available robotics solution to perform eight fusions simultaneously and carry out downstream tissue culture. Eighty antigens were immunized into eight mice, ten in each animal (Table 3a, b). The immunization protocol was deliberately kept short to ensure broad-spectrum immunoreactivity and decrease immunofocussing and immunodominance [11]. After the primary immunization, the animals were boosted only once, on day 14, and the spleens were harvested on day 18. The fusion was robotically accomplished using standard protocols and the culture supernatants were subsequently harvested and screened by AMA. As shown in Table 3c, monoclonal antibodies were raised against 67 of the 80 antigens (83%) as tested by AMA. IgM secreting clones were raised against 32 (40%) of these antigens and we detected IgG secreting clones against 62 (77.5%) of the target antigens. Only IgG secretors were tested in further assays, and of these, hybridoma cell lines were isolated generating antibodies against 32 (40%) target antigens that were positive in at least one further immunoassay. Cell lines against

Table 3. 80-antigen pilot test. a) Antigens details. Details of the antigens used for this immunisation experiments are here described. DWIR: "Data Withheld at Investigators Request" b) Individual results of the 80-antigen pilot test. Individual results are shown. For each of the 80 antigens used in the trial, the total number of specific hybridomas selected, their isotype and the number of these that showed ELISA positivity are listed. c) Summary and analysis of 80-antigen test results. All selected IgG species were screened by ELISA and Western blot. BlockA shows the number of antigens for which at least one mAb was detected by AMA and the number of ELISA and Western blot positives. Block B represents the breakdown of the results: the number of antigens for which antibodies were shown to be positive by AMA, ELISA and Western blot, by AMA and ELISA, by AMA and Western blot or by AMA only. Antigens for which no antibodies were selected or for which no Western blot was performed are shown in the two last columns of the table.

3a:

Antigen name	Owner	Protein's full name	Length of full protein	Size of antigen (kD or aa)	Antigen origin
NK p46 D2	Dr. Mandelboim	NKp46	50 kDa	50 kDa	
NK p30	Dr. Mandelboim	NKp30	30 kDa	50 kDa	
NK AT8	Dr. Mandelboim	KIR2DS4	50 kDa	70 kDa	
DN 26	Dr. Averof	Af-apterous	unknown	280 aa	*A. franciscana*
Reverse gyrase	Dr. Stock	Reverse Gyrase	120 kDa	120 kDa	*A. fulgidus*
hGH	DWIR				
hGA	DWIR				
GST-Snu 13	DWIR				
GST-TgS1	DWIR				
GST-GRIP1	DWIR				
Vac ATPase	Dr. Stock	Vacuolar H+-ATPase	650 kDa (9 subunits: 64, 53, 35, 25, 20, 11, 13, 73 (TM), 8 (TM) kDa)	650 kDa	*T. thermophilus*
Smoothened	Dr. Therond	Smoothened	1031 aa	33 kDa	*D. melanogaster*
Hedgehog	Dr. Therond	Hedgehog	472 aa	25 kDa	*D. melanogaster*
CG33206A	Dr. Therond	D-GMAP210	1398 aa	302 aa	*D. melanogaster*
CG33206B	Dr. Therond	D-GMAP210	1398 aa	307 aa	*D. melanogaster*
Tace DOH CyT	DWIR				
GST-Tip 60	DWIR				
Grip1	DWIR				
GST-Hog1	DWIR				
CJ0601C	Dr. Lykke-Møller Sørensen	CJ0601c	49.7 kDa	49.7 kDa	*C. jejuni*
GST-Cofilin1 muscle	DWIR				
Gelsolin	DWIR				
Profilin2	DWIR				
12,6	DWIR				
RAC 1 V-12	DWIR				
Rho AV 14	DWIR				
Cdc42 Hs wt	DWIR				
Rab 27	Dr. Christoforidis	Rab27a	221 aa	221 aa	*H. sapiens*
Past 1/EDH	DWIR				
GST ADF	DWIR				
CHE alpha	DWIR				
Stat 1TC	DWIR				
CLIP 170 H2	DWIR				
PIST	Dr. Barr	PIST/GOPc	463 aa	475 aa	*H. sapiens*
SEC 18P	Dr. Ungermann	Sec18	66 kDa	66 kDa	*S. cerevisiae*
Yed Z-His	Dr. De Gier	YedZ	219 aa	211 aa	*E. coli*
CES1	DWIR				
DMAp	Dr. Averof	apterous	496 aa	60 aa	*D. melanogaster*
PA	DWIR				
SS	DWIR				

Table 3. 3a: Continued

Antigen name	Owner	Protein's full name	Length of full protein	Size of antigen (kD or aa)	Antigen origin
PopD	Dr. Dessen	PopD	295 aa	295 aa	*P. aeruginosa*
PcrV	Dr. Dessen	PcrV	294 aa	294 aa	*P. aeruginosa*
YID alpha sup12	DWIR				
ES1	Dr. Tocchini-Valentini	TRNA splicing endonuclease	182 aa	20 kDA	*S. solfataricus*
GST	DWIR				
POP	DWIR				
Profilin 1	DWIR				
FLN	Dr. Mosialos	Folliculin isoform 2	342 aa	62.7 kDa	*H. sapiens*
IK Beta	DWIR				
P3 fragment	Dr. Fankhauser	PIF4 (bHLH009)	430 aa	36.1 kDa	*A. thaliana*
GST cofilin2 non-muscle	DWIR				
SCAMP	DWIR				
EB-1	Dr. John	EB1	30 kDa	30 kDa	*H. sapiens*
UNC 59–61	Dr. John	Unc59, Unc61 complex	53 kDa each, tetrameric	53 kDa each	*C. elegans*
2C1	DWIR				
HIWI2	DWIR				
HILI	DWIR				
DNX	DWIR				
FABP	DWIR				
FADD	Dr. Ruberti	FADD	23 kDa	23 kDa	*H. sapiens*
KOC-1	DWIR				
TKT-L1	DWIR				
FADD-DD	Dr. Ruberti	FADD Death Domain	13.5 kDa	13.5 kDa	*H. sapiens*
cKet B2	Dr. Bullard	CketB2 (expressed fragment of Sls)	2 MDa	313 aa	*D. melanogaster*
Ket35/1	Dr. Bullard	Ket35–1 (expressed fragment of Kettin)	500 kDa	379 aa	*D. melanogaster*
Ket35/2	Dr. Bullard	Ket35–2 (expressed fragment of Kettin)	500 kDa	816 aa	*D. melanogaster*
Tncf 1	Dr. Bullard	TroponinC-F1	18 kDa	18 kDa	*L. indicus*
GST Tip1	Dr. Brunner	Tip1p	461 aa	50 kDa	*S. pombe*
LUMA peptide	DWIR				
YKt6	Dr. Ungermann	Ykt6	25 kDa	140 aa	*S. cerevisiae*
SHARP-SPOC	DWIR				
RXRb-LBD	DWIR				
SPD 1	Dr. Glotzer	SPD1	443 aa	443 aa	*C. elegans*
ZEN 4	Dr. Glotzer	ZEN-4	775 aa	434 aa	*C. elegans*
14–3-3 epsilon	Prof. Cesareni	14.3.3 epsilon	255 aa	255 aa	*H. sapiens*
14–3-3 zeta	Prof. Cesareni	14.3.3 zeta	245 aa	245 aa	*H. sapiens*
14–3-3 beta	Prof. Cesareni	14.3.3 beta	245 aa	245 aa	*H. sapiens*
P63 C-term	Prof. Cesareni	p63	641 aa	42 kDa	*H. sapiens*
POB 1	Prof. Cesareni	POB	511 aa	98 aa	*H. sapiens*
GST-Hrs	Prof. Cesareni	HRS	777 aa	280 kDa	*H. sapiens*

Table 3.

3b:

Mouse	Antigen name	Total AMA positives	IgM positives (AMA)	IgG positives (AMA)	IgG positives (ELISA)
9721	NK p46 D2	6	4	2	1
	NK p30	13	7	6	2
	NK AT8	1	0	1	1
	DN 26	2	0	2	0
	Reverse gyrase	4	0	4	4
	hGH	2	0	2	0
	hGA	0	0	0	0
	GST-Snu 13	2	0	2	1
	GST-TgS1	1	1	0	0
	GST-GRIP1	1	1	0	0
9715	Vac ATPase	2	0	2	0
	Smoothened	0	0	0	0
	Hedgehog	0	0	0	0
	CG33206A	6	5	1	0
	CG33206B	4	0	4	4
	Tace DOH CyT	3	0	3	0
	GST-Tip 60	7	1	6	6
	Grip1	4	0	4	4
	GST-Hog1	0	0	0	0
	CJ0601C	0	0	0	0
9717	GST-Cofilin1 muscle	2	0	2	0
	Gelsolin	5	3	2	0
	Profilin2	2	1	1	0
	12,6	8	0	8	8
	RAC 1 V-12	3	0	3	0
	Rho AV 14	2	2	0	0
	Cdc42 Hs wt	7	3	4	1
	Rab 27	3	0	3	0
	Past 1/EDH	8	4	4	0
	GST ADF	5	2	3	0
9714	CHE alpha	2	1	1	0
	Stat 1TC	3	2	1	0
	CLIP 170 H2	0	0	0	0
	PIST	1	0	1	1
	SEC 18P	3	0	3	2
	Yed Z-His	12	0	12	0
	CES1	8	4	4	1
	DMAp	4	3	1	0
	PA	2	1	1	0
	SS	4	1	3	2
9716	PopD	12	0	12	10
	PcrV	2	1	1	0
	YID alpha sup12	10	0	10	8
	ES1	1	0	1	0
	GST	1	0	1	0
	POP	1	0	1	0
	Profilin 1	2	0	2	0
	FLN	0	0	0	0
	IK Beta	3	0	3	3
	P3 fragment	0	0	0	0
9718	GST cofilin2 non-muscle	2	0	2	0
	SCAMP	0	0	0	0
	EB-1	0	0	0	0
	UNC 59–61	5	0	5	0
	2C1	4	0	4	0

Table 3. 3b: Continued

Mouse	Antigen name	Total AMA positives	IgM positives (AMA)	IgG positives (AMA)	IgG positives (ELISA)
	HIWI2	5	3	2	0
	HILI	4	1	3	0
	DNX	5	0	5	0
	FABP	4	2	2	0
	FADD	16	5	11	5
9719	KOC-1	8	5	3	0
	TKT-L1	3	1	2	0
	FADD-DD	0	0	0	0
	cKet B2	9	1	8	8
	Ket35/1	4	1	3	0
	Ket35/2	1	0	1	0
	Tncf 1	10	5	5	0
	GST Tip1	4	1	3	1
	LUMA peptide	2	0	2	1
	YKt6	2	0	2	2
9720	SHARP-SPOC	4	1	3	0
	RXRb-LBD	1	0	1	0
	SPD 1	0	0	0	0
	ZEN 4	7	2	5	4
	14–3-3 epsilon	7	1	6	0
	14–3-3 zeta	4	0	4	0
	14–3-3 beta	2	0	2	0
	P63 C-term	0	0	0	0
	POB 1	1	1	0	0
	GST-Hrs	1	1	0	0

3c:

BLOCK A					BLOCK B					
AMA positives	AMA IgM	AMA IgG	ELISA positives	WB positives	AMA/ ELISA/WB positives	AMA/ ELISA positives	AMA/WB positives	AMA only positives	Nega- tives	Missing WB data
9	4	7	5	2	2	3	0	4	1	2
6	2	6	3	1	1	2	0	3	4	0
10	6	8	2	2	2	0	0	8	0	3
9	6	9	4	5	4	0	1	4	1	1
8	1	8	5	6	3	2	3	0	2	2
8	4	8	1	4	1	0	3	4	2	3
9	6	9	4	1	1	3	0	5	1	4
8	5	6	1	1	1	0	0	7	2	0
67	34	61	25	22	15	10	7	35	13	15

15 of the 80 targets produced antibodies that were positive by both western blot and ELISA; 10 were positive by AMA and ELISA only, and seven positive by AMA and western blot only.

4 Discussion

We have presented a novel method for the high throughput production of mouse-derived monoclonal antibodies using multiplexed immunizations and a highly sensitive and parallel screening protocol based on antigen-coated microarrays. We have shown that it is possible to obtain specific monoclonal antibodies against each of the five antigens used for the multiplexing immunisation trial, and these observations were validated by several tests performed using different antigens. The novel screening method, which represents a major advance of the antibody production platform, allows for a quick and highly sensitive analysis of vast hybridoma libraries.

The disparity in the correlation between AMA and ELISA is due to two factors. The first factor is that the sensitivity of the AMA is higher than that of the ELISA

(Tonkinson, J.L, http://www.devicelink.com/ivdt/archive/03/03/001.html) and that the threshold for what is considered positive by AMA was set too low. Indeed, when the threshold was increased the correlation between AMA and ELISA approached 100% (data not shown). The higher sensitivity of the AMA means also that factors such as low antibody concentration in the supernatant or the presence of an antibody of low binding affinity would give a positive result by AMA that would be negative by ELISA. Secondly, the conformation of the proteins on the different substrates could have been sufficiently different that antibody-binding sites on the protein that are available on the AMA substrate are masked by adsorbing the protein onto the polycarbonate substrate in the ELISA. Indeed this is borne out by the fact that seven of the antigens used in the 80-antigen trial (PcrV, ES1, GST, UNC59–61, HILI, HIWI, Yed-Z-His) were negative by ELISA and subsequently positive by western blot. It is also necessary to mention that the immunization protocol was shorter for the 80-antigen trial and the supernatants were harvested earlier compared to the experiments shown in Tables 1 and 2. We speculate that several species of monoclonal antibodies showing either lower affinity or lower concentrations were produced and thus fell below the threshold of detection for the ELISA.

Furthermore, AMA represents a qualitative improvement over ELISA as it allows for the selection of clones secreting antibodies with different isotypes directly at the primary screen using a mixture of up to five isotype specific secondary antibodies, each with a different flurochrome. ELISA can also be used to determine the isotype of monoclonal antibodies but this is however more laborious requiring up to five separate ELISAs per hybridoma (IgM, IgG1, IgG2a, IgG2b, IgG3). IgM isotypes are often more useful to investigators for fluorescence-based assays such as FACS analysis or immunofluorescence, but this isotype is almost useless for assays involving protein-A and protein-G as detection reagents, most notably immuno-electronmicroscopy and immunoprecipitation, due to their lack of affinity for protein-A or protein-G. IgM isotype antibodies are also notoriously difficult to purify and in these two cases IgG isotypes are more desirable.

In the 80-antigen pilot study we achieved a success rate of 40% for antibodies that worked in at least one other immunoassay, or 28% for antibodies which were functional in at least one immuno-application. Comparison with similar studies is difficult, as others have focused on the production of antibody serum titre [12–14] as opposed to the isolation and production of hybridomas, however when compared to the best of these serum-titre studies [12], our method is about half as successful as the 80% success rate achieved there.

Improvements in these success rates may be possible by slightly lengthening the immunization protocol. The longer immunization protocols used in the experiments represented by Tables 1 and 2 may well account for the higher efficacy over the 80-antigen experiment. It is also possible that increasing the number of immunogens for each animal may ultimately lower the number of positive clones in any one fusion. Indeed, subsequent production runs within our laboratory have shown that five antigens per animal is optimal, however recent improvements in immunization strategies have allowed us to immunize using ten antigens with higher success rate than in the 80-antigen experiment (data not shown). Using genetic immunization protocols may prove even more effective in attaining a suitable response against a higher proportion of the target antigens [12].

In summary, we have developed a fast, economical, high-throughput method for hybridoma generation, which improves upon the conventional, non-automated system in the following ways. Firstly, the turnaround time from receipt of the antigen to delivery of cells to the investigator has been reduced from 4 to 6 months to around 2 months. Secondly, there is a cost reduction of approximately five-fold (in the case of five antigens per animal) per target. Thirdly, the throughput has increased from approximately 20 specific target antigens per capita per annum to 150 specific target antigens per capita per annum. Further increases in throughput would be possible by automating the downstream tissue culture (including clonal expansion and freeze down steps), a bottleneck which was not addressed in this study. A simple scaling up of the methodology proposed here along with the above-mentioned automation of the clonal expansion and freeze down, could help to alleviate the current restrictions on the availability of these important affinity reagents.

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5 References

[1] Phelan, M. L., Nock, S., *Proteomics* 2003, *3*, 2123–2134.

[2] Nord, K. *et al.*, *Nat. Biotechnol.* 1997, *15*, 772–777.

[3] Knappik, A. *et al.*, *J. Mol. Biol.* 2000, *296*, 57–86.

[4] Li, M., *Nat. Biotechnol.* 2000, *18*, 1251–1256.

[5] Bock, L. C., Griffin, L. C., Latham, J. A., Vermaas, E. H., Toole, J. J., *Nature* 1992, *355,* 564–566.

[6] D'Mello, F., Howard, C. R., *J. Immunol. Methods* 2001, *247,* 191–203.

[7] Irving, R. A., Kortt, A. A., Hudson, P. J., *Immunotechnology* 1996, *2,* 127–143.

[8] Gram, H. *et al., Proc. Natl. Acad. Sci.* 1992, *89,* 3576–3580.

[9] Krieg, A. M. *et al., Nature* 1995, *374,* 546–549.

[10] Kohler, G., Milstein, C., *Nature* 1975, *256,* 495–497.

[11] Harlow, E., Lane, D., *ANTIBODIES: A Laboratory Manual.* Cold Spring Harbor Laboratory, New York 1998.

[12] Chambers, R. S., Johnston, S. A., *Nat. Biotechnol.* 2003, *21,* 1088–1092.

[13] Field, C. M., Oegema, K., Zheng, Y., Mitchison, T. J., Walczak, C. E., *Methods Enzymol.* 1998, *298,* 525–554.

[14] Pizza, M. *et al., Science* 2000, *287,* 1816–1820.

[15] Vinkemeier, U. *et al., EMBO J.* 1996, *15,* 5616–5626.

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High-level generation of polyclonal antibodies by genetic immunization

Ross S Chambers & Stephen Albert Johnston

Antibodies are important tools for investigating the proteome, but current methods for producing them have become a rate-limiting step[1]. A primary obstacle in most methods for generating antibodies or antibody-like molecules is the requirement for at least microgram quantities of purified protein. We have developed a technology for producing antibodies using genetic immunization[2]. Genetic immunization–based antibody production offers several advantages, including high throughput[3] and high specificity. Moreover, antibodies produced from genetically immunized animals are more likely to recognize the native protein[2]. Here we show that a genetic immunization–based system can be used to efficiently raise useful antibodies to a wide range of antigens. We accomplished this by linking the antigen gene to various elements that enhance antigenicity and by codelivering plasmids encoding genetic adjuvants. Our system, which was tested by immunizing mice with >130 antigens, has shown a final success rate of 84%.

Genetic immunization has received relatively little attention as a method for producing antibodies for proteomic applications. One reason has been the variable success of genetic immunization in producing antibodies[4]. To develop improved genetic immunization plasmids for more reliable generation of antibodies, we began with plasmid pBQAP10 (Fig. 1), which encodes a secretion leader sequence from the highly expressed human gene encoding α1-antitrypsin (AAT). Many studies have shown that adding a secretion leader sequence can markedly increase the antibody response[5,6]. After the leader sequence is a unique 20–amino acid antigenic tag that we included as an internal control. Secretion of the antigen may be blocked by 'quality control' if it is poorly folded or insoluble[7]. To improve protein solubility, we included a highly soluble and stably folded domain from the rat cartilage oligomerization matrix protein (COMP)[8]. The 46-residue COMP domain can also form pentamers and may enhance antigen uptake by antigen-presenting cells or allow T helper–independent B-cell activation[9,10].

We used the human gene encoding AAT as an antigen to test the efficacy of pBQAP10 in genetic immunization. Many different cytokines had earlier been tested as genetic adjuvants, with mixed results[11]. Plasmids expressing granulocyte/macrophage colony-stimulating factor (GM-CSF) have been widely used in genetic immunization studies and almost always result in an increase in antibody titer[11]. GM-CSF is a potent growth factor for dendritic cells, although its exact mechanism of action in genetic immunization is poorly understood. We immunized mice with the AAT-expressing plasmid using a gene gun, either with or without coadministration of plasmids encoding the cytokines GM-CSF and FMS-like tyrosine kinase 3 ligand (Flt3L)[12]. ELISA measurements of sera showed that the mice coimmunized with both the GM-CSF and Flt3L plasmids had approximately a ninefold higher antibody titer (3×10^4 titer, Fig. 2a). For comparison, a group of mice immunized conventionally using AAT protein with Freund's complete adjuvant produced antibody titers of 7×10^4. All genetically immunized mice responded with relatively little variation in titers (Fig. 2b). Isotyping of the AAT antibodies showed only the IgG_1 isotype (data not shown). The specificity of the sera was tested by probing a western blot containing AAT mixed with an *Escherichia coli* whole-cell extract. Pooled sera from five mice recognized a single band of the correct size for AAT (Fig. 2c).

To evaluate the general usefulness of this antibody production system, we tested it using a set of 100 antigen genes (see Supplementary Table 1 online). Of the 100 genes tested, 36% encoded fragments of the mature form of the protein. The average identity of the human antigens to mouse proteins was 76%, and the average antigen size was 179 residues. Most of the genes were of human origin, and we explored three general sources of antigen genes: genomic DNA (20 genes), cDNA (52) and gene synthesis from oligonucleotides (28). In principle, amplifying genes from genomic DNA is the simplest approach because only a single template and two PCR primers are required per gene, or four primers for nested PCR. Genes fragmented into small exons may present a problem. For example, genes in the human genome are on average broken into 8.8 exons encoding an average length of 50 residues[13]. Using cDNA would bypass this problem but is more difficult logistically. Both genomic DNA and cDNA have the disadvantage that the genes may contain suboptimal codon usage. Codon optimization of genes has been shown to markedly increase translation and, as a consequence, antibody responses[14,15]. Gene synthesis allows codons to be optimized for expression and gives unrestricted access to any gene sequence. We recoded genes using a subset of codons allowing efficient expression in both mice and *E. coli* (see Methods).

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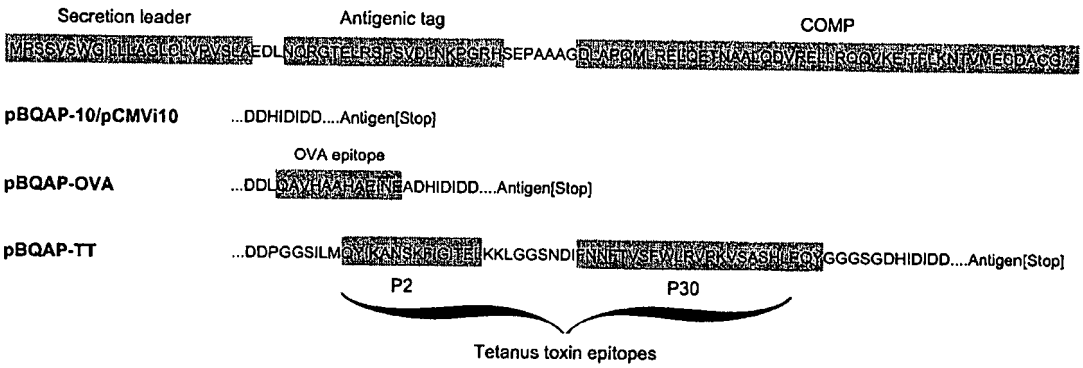

Figure 1 Design of genetic immunization vector. The plasmids pBQAP10, pBQAP-OVA and pBQAP-TT all contained the SP72 promoter and the rabbit β-globin terminator flanking the expression cassette shown above. The pCMVi10 plasmid is identical to pBQAP10 except that it contains the CMV promoter. The sequence HIDIDD is encoded by the 5′ flanks included in the PCR primers used to amplify the antigen gene.

PCR products of the 100 antigen genes were generated using primers with a flanking sequence containing deoxyuracil (dU) residues allowing rapid cloning[16]. The genes were cloned into either pBQAP10 (80 genes) or pCMVi10 (20) to allow genetic immunization of mice and into pGST-FRP for overexpression in *E. coli*. Overexpression was obtained successfully in *E. coli* with 88 of the 100 proteins. Groups of two CD1 mice were immunized and were boosted every 3 weeks until a total of four shots had been administered. Sera from mice were tested every 3 weeks by western blotting and were scored successful if 50 ng of the antigen was detected at sera dilutions of 1:5,000. Antibodies were detected against 62 of the 88 test antigens (70%) and were produced after an average of two immunizations (Supplementary Table 1 and Supplementary Fig. 1 online). The pBQAP10 and pCMVi10 vectors had similar efficacies.

Antigens that have high identity to sequences from the immunized host typically do not produce an antibody response as a result of tolerance mechanisms[17]. Analysis of the antigens tested in pBQAP10 and pCMVi10 indicated that this may indeed be a limitation, because antigens that failed to produce an antibody response had on average a higher identity to a mouse protein than successful antigens (69% versus 61%; Supplementary Table 1). Humoral tolerance can be overcome by adding exogenous T-cell epitopes fused to the antigen[18,19]. To evaluate this idea we created two new vectors, pBQAP-TT and pBQAP-OVA (Fig. 1), that contained either the P2 and P30 'universal' T-cell epitopes and flanking regions from tetanus toxin (50 residues) or the ovalbumin(325–336) T-cell epitope (12 residues).

A set of 38 gene fragments was cloned into either pBQAP-TT or pBQAP-OVA (see Supplementary Table 2 online). Most of the genes encoded proteins that were expected to be poorly antigenic, because they were small (≤20 amino acids), were highly identical to mouse sequences (≤100%) or had earlier failed using protein-based immunizations. In addition, we included five genes that had earlier failed to yield antibodies in genetic immunizations when cloned in pBQAP10. The target region of each gene was selected on the basis of its antigenicity index score[20]. On average, the antigens contained 73 amino acids and had a 90% identity to a mouse protein.

Protein was successfully overproduced in *E. coli* for 97% of the genes. Antibodies were produced after an average of two immunizations. Antigens identical to mouse sequences were as successful as antigens with lower identity, and there was no substantial difference in success rate between the two T-cell epitope vectors. Others have reported producing antibodies against self-proteins by fusing T-cell epitopes[18,19], and we have shown that this approach seems to work with many self-proteins. Four of the five antigens that earlier failed to induce antibodies in pBQAP10 now produced antibodies. Furthermore, four antigens that earlier failed to produce antibodies when delivered as protein now produced antibodies (ApoAV, R26W, RYR2, Ub). Overall, 87% of large antigens (≥70 residues) and 79% of the small antigens (≤20 residues) produced antibodies, with an overall success rate of 84% (Supplementary Table 2 and Supplementary Fig. 1).

There are few published studies with which the antibody production method developed in this study can be compared. The largest study to date is one that used protein immunizations with 570 antigens from *Neisseria meningitidis*[21]. Only 350 of the proteins could be overexpressed in *E. coli*, and of those, only 85 (24%) produced "strongly positive" antibodies. Another large study with a set of 40 synthetic peptides linked to keyhole limpet hemocyanin obtained a 63% success rate[22].

To investigate possible causes of failure in our system, we tested sera for antibodies against the antigenic tag. All of eight sera with antibodies against the test antigen also contained antibodies against the tag. Of ten sera that did not contain antibodies against the test antigen, eight did contain antibodies against the tag. Therefore, we can eliminate many nonimmunological causes of antibody response failure such as suboptimal bullet preparation, plasmid delivery, protein translation and protein secretion. Remaining possible causes of failure include post-translational modification of the antigen, structural features of the antigen and B-cell unresponsiveness. Sera were also tested for antibodies against other regions of the scaffold. We did not detect antibodies to the COMP domain or to the tetanus toxin epitopes, and only one of seven samples had antibodies against the ovalbumin epitope (data not shown).

To examine whether the antibodies we produced were useful for measuring the natural antigen, we used 12 of the antibodies to probe biological samples in which the antigen was known to be expressed. All 12 antibodies detected a protein of the correct size in the appropriate sample, but not in a control sample (Fig. 3). Sensitivity was tested with randomly selected antibodies by titrating the corresponding glutathione *S*-transferase (GST) fusion proteins on a western blot. Most of the antibodies, including those raised against self-proteins, could detect as little as a few nanograms of the GST protein (Supplementary Fig. 2).

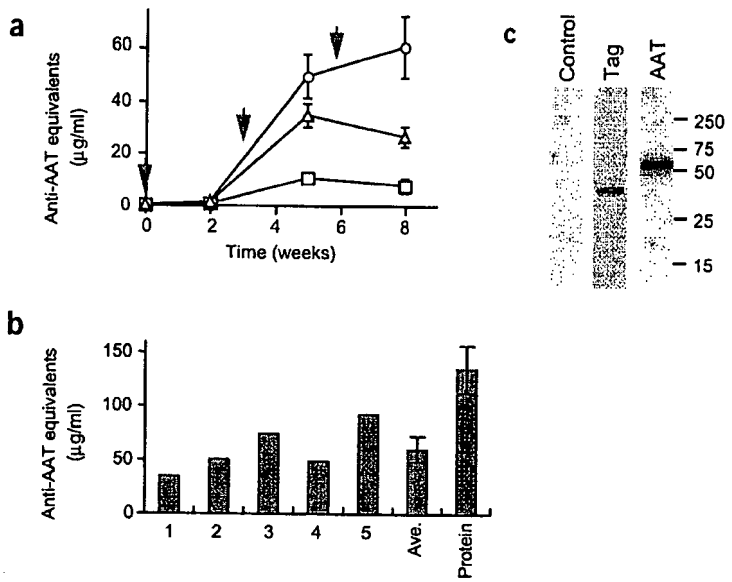

Figure 2 Antibody responses of mice immunized with pBQAP10-AAT. (a) Groups of five BALB/c mice were immunized with pBQAP10-AAT alone (□), with a GM-CSF plasmid (△) or with both GM-CSF and Flt3L plasmids (○). Antibodies to AAT were measured by ELISA and converted to monoclonal antibody equivalents using an anti-AAT monoclonal antibody of known concentration. The slopes of the curves for dilutions of the sera and the antibody were similar. Sera were diluted 1:250, 1:250, 1:1,000 and 1:6,000 for the 0, 2-, 5- and 8-week samples, respectively. Arrows indicate immunizations and bars standard errors. (b) Individual antibody concentrations measured by ELISA for five mice immunized three times with the plasmids containing genes encoding AAT, GM-CSF and Flt3L (AVE., average for these five mice) and a group of five mice immunized once with AAT protein. (c) Western blot analysis of sera pooled from five mice immunized as described in a. Control lane contains 10 μg of a whole-cell extract from *E. coli* with 50 ng of a GST fusion protein unrelated to AAT. The AAT and tag lanes are the same as the control lane, except for the addition to the samples before electrophoresis of 50 ng of pure AAT and 50 ng of GST tag, respectively. Sera were diluted 1:5,000.

Although antibodies were obtained against ≤84% of the gene products that could be expressed in *E. coli*, a number of caveats should be mentioned. First, protein synthesis in at least one system is required to test these antibodies. Although the proteins do not have to be purified, a great advantage over alternative methods, they do have to be made, because specificity cannot be confirmed without a protein source. If this is considered, the success rate is somewhat reduced to 82% for the small, difficult antigens expressed with T-cell epitopes, and 62% for the antigens expressed without the T-cell epitope. Overall, 90% of the 133 different antigens were successfully overexpressed in *E. coli*. This is a higher success rate than reported by other large-scale expression studies[21,23]. This higher success rate may largely be attributed to selecting small soluble fragments of proteins as well as avoiding membrane proteins or at least the membrane-associating region. Membrane proteins are typically the most difficult to overexpress, and it should be noted that half of the proteins that we failed to express in *E. coli* were membrane proteins. Moreover, 21% of the sera (Supplementary Fig. 1) showed some cross-reactivity with unexpected proteins in *E. coli* extracts supplemented with an irrelevant GST fusion protein. There is no indication that these sera will react with antigens from the same organism as the one used for genetic immunization; however, this finding shows a relatively high rate of spurious cross-reaction, which should always be borne in mind when testing these, or indeed any polyclonal, sera.

High-throughput genomic technologies currently produce complete genome sequences and allow the measurement of entire mRNA populations. Although these innovations have revolutionized biology, their impact will be limited unless the information generated can be translated to the protein level in a correspondingly high-throughput manner. We have developed a high-throughput system for generating antibodies that can help close the gap. Application of this system could range from small-scale analysis of interesting gene sets discovered by microarray analysis, to systematic generation of antibodies against all putative proteins discovered in genome sequencing projects. Each CD1 mouse yields ≤2 ml of serum, sufficient for hundreds of immunoassays. Spleens from the mice can be saved, allowing larger amounts of highly valuable antibodies to be generated later as monoclonal or single-chain antibodies[24,25].

METHODS

Construction of plasmids. The genetic immunization plasmids were derived from pCAGGS[26]. We replaced the human cytomegalovirus (CMV) promoter with a synthetic promoter, SP72. The SP72 element was designed *de novo* from consensus binding sites for transcription factors and rivals CMV in terms of producing antibody responses (B. Qu, University of Texas-Southwestern Medical Center, Dallas, Texas, USA, personal communication). A 618-bp fragment containing the SP72 promoter was subcloned at the *Sal*I and *Eco*RI sites, replacing the CMV promoter and intron, to create pSP72. Gene synthesis was used to construct a 346-bp DNA fragment containing, in the following order, an *Eco*RI site, a consensus translation initiation site, the leader sequence from *AAT*, the antigenic tag, COMP and restriction sites for *Bcl*I, *Xma*I and *Xba*I. The fragment was digested with *Eco*RI and *Xba*I and subcloned into the same sites in pSP72 to create pBQAP10. The plasmid pCMVi10 was identical except that it retained the original CMV promoter and intron. The plasmids pBQAP-OVA and pBQAP-TT were based on pBQAP10 and were created by subcloning a *Bgl*II- and *Xma*I-digested DNA fragment encoding the T-cell epitopes, and created by gene synthesis, into the *Bcl*I and *Xma*I sites. A new *Bcl*I site was designed after the T-cell epitope coding regions. The plasmid pGST-FRP was derived from pGST-CS[27] by subcloning a pair of annealed oligonucleotides at the *Nco*I and *Eco*RI sites. This replaced the existing multiple cloning sites for *Bgl*II, *Bam*HI and *Xma*I. The expression plasmids encoding GM-CSF and Flt3L were constructed by subcloning mouse cDNAs into pCMVi-SS[28] at the *Bgl*II and *Kpn*I sites.

Gene synthesis. Genes were designed with a set of codons selected for efficient expression in both mice and *E. coli*, and for design flexibility to avoid hairpins and other inappropriate matches among the sequence that can hinder gene synthesis. The codons used were as follow: Ala, GCA (33%), GCT (33%), GCC (34%); Cys, TGT (50%), TGC (50%); Asp, GAT (50%), GAC (50%); Glu, GAG (50%), GAA (50%); Phe, TTT (25%), TTC (75%); Gly, GGT (50%), GGC (50%); His, CAT (25%), CAC (75%); Ile, ATT (25%), ATC (75%); Lys, AAG (50%), AAA (50%); Leu, CTG (100%); Met, ATG (100%); Asn, AAC (100%); Pro, CCG (50%), CCA (50%); Gln, CAG (75%), CAA (25%); Arg, CGT (25%), CGC (75%); Ser, TCT (50%), AGC (50%); Thr, ACT (50%), ACC (50%); Val, GTG (75%), GTT (25%); Trp, TGG (100%); Tyr, TAT (50%), TAC (50%). A set

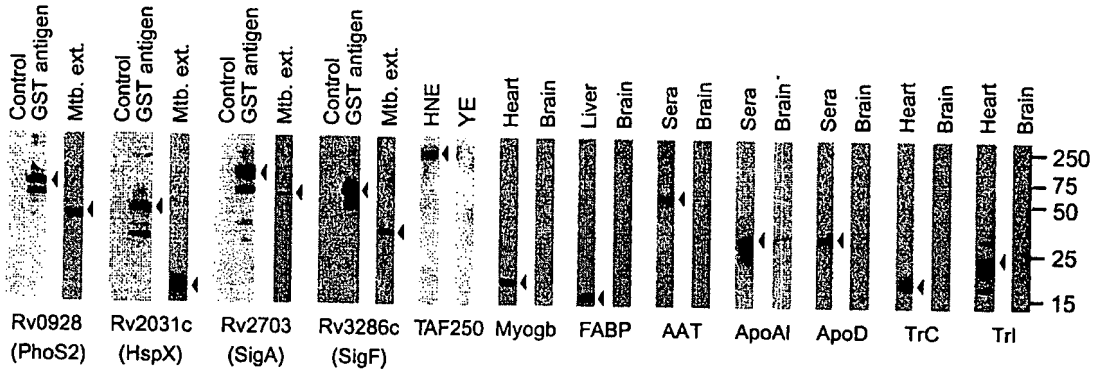

Figure 3 Western blot analysis of natural extracts. All antibodies were diluted 1:1,000. The antibodies raised against the Mtb proteins were used to probe western blots containing 3.25 μg of a *Mycobacterium tuberculosis* whole-cell extract (Mtb. ext.). As a control, the antibodies were used to probe a western blot containing 10 μg of an *E. coli* whole-cell extract with either 50 ng of an unrelated GST fusion protein (control) or the relevant GST antigen. The TAF250 antibody was probed against 4.5 μg of a HeLa cell nuclear extract (HNE) or 6 μg of a yeast extract (YE). The AAT, ApoAI and ApoD antibodies were probed against 7 μg of human sera or, as a control, 25 μg of a human brain extract. The myoglobin, FABP, TrC and Trl antibodies were probed against 25 μg of human brain, liver or heart extract. Arrows indicate the known sizes of the mature proteins.

of overlapping oligonucleotides was designed using the custom software DNABuilder (http://cbi.swmed.edu/computation/cbu). The oligonucleotides were assembled into a DNA fragment using PCR[29]. Genes were subcloned into the appropriate plasmids and sequenced to identify a correct clone. Mutations occurred at a frequency of 0.3%.

UDG cloning. PCR products were generated using primers containing 5′ flanks as described[16]. The forward primers contained the flanking sequence 5′-ATAUCGAUAUCGAUGAU-3′, and the reverse primers contained the flanking sequence 5′-AGUGAUCGAUGCATUACU-3′. Vector preparations were created by digesting the plasmids with *BclI* and *XmaI* (pBQAP10, pBQAP-OVA, pBQAP-TT) or *BglII* and *XmaI* (pGST-FRP), and ligating the following oligonucleotides to the 4-bp overhangs: 5′-GATCATATCGATATCGATGAT-3′ and 5′-CCGGAGTGATCGATGCATTACT-3′. PCR products were subcloned by mixing 50 ng of the vector preparation with 10 ng of the PCR product in the presence of 0.5 units of uracil DNA glycosylase (New England Biolabs), 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol in a final volume of 10 μl. Reactions were incubated at 37 °C for 30 min, and 1 μl was used to transform *E. coli* DH10B.

Genetic immunization and analyses. All procedures for handling mice were approved by the University of Texas Southwestern Medical Center IACRAC. Plasmids were delivered using the Helios gene gun (Bio-Rad). Bullets were prepared according to the manufacturer's instructions with a mixture of plasmids encoding the antigen and plasmids encoding mouse GM-CSF and mouse Flt3L (2:1:1 ratio). Each bullet contained ~1 μg of DNA. Mice were anesthetized with avertin (0.4 ml/20 g mouse) and shot in each ear using 400 p.s.i. to fire the gene gun. Blood was collected by tail bleeds and allowed to stand for 2 h at 24 °C, after which the sera were collected by centrifugation. Western blots and ELISAs were done as described[3]. Each ELISA was done using an AAT monoclonal antibody as a standard (Calbiochem) to calculate antibody equivalents in micrograms per milliliter. Titers were defined as the reciprocal of the sera dilution that produced a signal twofold above background (age-matched sera). GST fusion proteins were generated in *E. coli* strain DH10B by inducing 2-ml log-phase cultures with isopropyl-β-D-thiogalactoside. Whole-cell extracts were prepared from bacteria 2 h after induction. Cells were pelleted, resuspended in 200 μl of PBS, mixed with 200 μl of SDS lysis buffer and heated for 5 min at 95 °C.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests

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1. Kodadek, T. Protein microarrays: prospects and problems. *Chem. Biol.* 8, 105–115 (2001).
2. Tang, D.-C., DeVit, M. & Johnston, S.A. Genetic immunization is a simple method for eliciting an immune response. *Nature* 356, 152–154 (1992).
3. Sykes, K.F. & Johnston, S.A. Linear expression elements: a rapid, *in vivo*, method to screen for gene functions. *Nat. Biotechnol.* 17, 355–359 (1999).
4. Babiuk, L.A., van Drunen Littel-van den Hurk, S. & Babiuk, S.L. Immunization of animals: from DNA to the dinner plate. *Vet. Immunol. Immunopathol.* 72, 189–202 (1999).
5. Svanholm, C., Bandholtz, L., Lobell, A. & Wigzell, H. Enhancement of antibody responses by DNA immunization using expression vectors mediating efficient antigen secretion. *J. Immunol. Methods* 228, 121–130 (1999).
6. Li, Z. *et al.* Immunogenicity of DNA vaccines expressing tuberculosis proteins fused to tissue plasminogen activator signal sequences. *Infect. Immun.* 67, 4780–4786 (1999).
7. Hammond, C. & Helenius, A. Quality control in the secretory pathway. *Curr. Opin. Cell Biol.* 7, 523–529 (1995).
8. Terskikh, A.V. *et al.* "Peptabody": a new type of high-avidity binding protein. *Proc. Natl. Acad. Sci. USA* 94, 1663–1668 (1997).
9. St. Clair, N., Shenoy, B., Jacob, L.D. & Margolin, A.L. Cross-linked protein crystals for vaccine delivery. *Proc. Natl. Acad. Sci. USA* 96, 9469–9474 (1999).
10. Valenzuela, P., Medina, A., Rutter, W.J., Ammerer, G. & Hall, B.D. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 298, 347–350 (1982).
11. Scheerlinck, J.-P.Y. Genetic adjuvants for DNA vaccines. *Vaccine* 19, 2647–2656 (2001).
12. Antonysamy, M.A. & Thomson, A.W. Flt3 ligand (FL) and its influence on immune reactivity. *Cytokine* 12, 87–100 (2000).
13. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. *Nature* 409, 860–921 (2001).
14. Andre, S. *et al.* Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J. Virol.* 72, 1497–1503 (1998).
15. Stratford, R. *et al.* Influence of codon usage on the immunogenicity of a DNA vaccine against tetanus. *Vaccine* 19, 810–815 (2001).
16. Smith, C., Day, P.J.R. & Walker, M.R. Generation of cohesive ends on PCR products by UDG-mediated excision of dU, and application for cloning into restriction digest-linearized vectors. *PCR Methods Appl.* 2, 328–332 (1993).
17. Zinkernagel, R.M. What is missing in immunology to understand immunity? *Nat. Immunol.* 1, 181–185 (2000).

18. King, C.A. *et al.* DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. *Nat. Med.* 4, 1281–1286 (1998).
19. Dalum, I., Jensen, M.R., Hindersson, P., Elsner, H.I. & Mouritsen, S. Breaking of B cell tolerance toward a highly conserved self protein. *J. Immunol.* **157**, 4796–4804 (1996).
20. Jameson, B.A. & Wolf, H. The antigenic index: a novel algorithm for predicting antigenic determinants. *Comput. Appl. Biosci.* 4, 181–186 (1988).
21. Pizza, M. *et al.* Identification of vaccine candidates against serotype B *Meningococcus* by whole-genome sequencing. *Science* **287**, 1816–1820 (2000).
22. Field, C.M., Oegema, K., Zheng, Y., Mitchison, T.J. & Walczak, C.E. Purification of cytoskeletal proteins using peptide antibodies. *Methods Enzymol.* **298**, 525–541 (1998).
23. Braun, P. *et al.* Proteome-scale purification of human proteins from bacteria. *Proc. Natl. Acad. Sci. USA* **99**, 2654–2659 (2002).
24. Barry, M.A., Barry, M.E. & Johnston, S.A. Production of monoclonal antibodies by genetic immunization. *Biotechniques* **16**, 616–619 (1994).
25. Chowdhury, P.S., Viner, J.L., Beers, R. & Pastan, I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. *Proc. Natl. Acad. Sci. USA* **95**, 669–674 (1998).
26. Niwa, H., Yamamura, K. & Miyazaki, J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199 (1991).
27. Chang, C. *et al.* The Gal4 activation domain binds sug2 protein, a proteosome component, *in vivo* and *in vitro*. *J. Biol. Chem.* **276**, 30956–30963 (2001).
28. Sykes, K.F. & Johnston, S.A. Genetic live vaccines mimic the antigenicity but not pathogenicity of live viruses. *DNA Cell Biol.* **18**, 521–531 (1999).
29. Stemmer, W.P.C., Crameri, A., Ha, K.D., Brennan, T.M. & Heyneker, H.L. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* **164**, 49–53 (1995).